AD_____

Award Number: DAMD17-01-1-0434

TITLE: Exploiting the Novel Repressed Transactivator Assay to
         Identify Protein Interactors and Peptide Inhibitors of
         the Myc Oncoprotein

PRINCIPAL INVESTIGATOR: Linda Z. Penn, Ph.D.

CONTRACTING ORGANIZATION: University Health Network
                           Toronto, Ontario M5G 2C4   Canada

REPORT DATE: August 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Annual (1 Aug 01 –31 Jul 02)

4. TITLE AND SUBTITLE	5. FUNDING NUMBERS
Exploiting the Novel Repressed Transactivator Assay to Identify Protein Interactors and Peptide Inhibitors of the Myc Oncoprotein	DAMD17-01-1-0434

6. AUTHOR(S)
Linda Z. Penn, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
University Health Network Toronto, Ontario M5G 2C4 Canada E-Mail: lpenn@uhnres.utoronto.ca	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSORING / MONITORING AGENCY REPORT NUMBER
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT	12b. DISTRIBUTION CODE
Approved for Public Release; Distribution Unlimited	

13. ABSTRACT (Maximum 200 Words)

none provided

14. SUBJECT TERMS
breast cancer, c-myc oncogene, breast carcinoma, novel two-hybrid, protein, protein interaction, peptide inhibitors, transformation, novel therapeutics

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Fundamental research clearly shows that the product of the c-myc oncogene is often expressed at elevated levels in a large number of breast cancers and is associated with poor prognosis, high-risk disease (for further detail, see our recent review (3). Indeed, Myc drives cell proliferation and can initiate as well as contribute to tumor development, as confirmed by both in vitro and in vivo models of breast cancer. The highly conserved regions of the Myc protein are thought to participate in protein:protein interactions. Importantly, blocking these sites can effectively inhibit Myc function and block breast carcinoma cell growth. Thus, Myc is a valid target for the development of novel therapeutics to inhibit mammary cells of malignant transformations in a specific and sensitive manner. This is the primary goal of this proposal.

To achieve our goal, we are using the novel Repressed Transactivator Assay (RTA) developed in collaboration with our lab (1). The RTA is an in vivo functional assay that will enable Myc-binding proteins and inhibitors of Myc:protein interactions to be identified and characterized. This experimental tool is conceptually similar to the conventional "two-hybrid" technology but offers significant improvement. First, the RTA allows transactivator bait proteins like Myc, to be used in a two-hybrid approach to clone novel interactors. Second, the ability of the RTA to identify inhibitors of a given protein-protein interaction makes it particularly well suited for high throughput screening of protein-protein interactions and the identification of inhibitor compounds.

Objective/hypothesis

Our hypothesis is that Myc is a valid target for the development of novel therapeutics aimed at blocking essential Myc:protein complexes that drive the carcinogenic process of mammary epithelial cells.

Specific Aims

1) We will use the RTA to identify the Myc-binding domain of a recently identified Myc-interactor, TRRAP (2), and delineate the nature of a "Myc-signature sequence" with which other Myc-binding proteins can be identified by sequence databank analysis.
2) Clone and characterize novel Myc-binding protein using the RTA to screen for MycNTD interactors in cells derived from human breast carcinomas.
3) With these experimental tools in hand we will then use the RTA to identify specific inhibitors of these protein:protein interactions that will block Myc function and inhibit tumor cell proliferation.

Body

With support from the DOD, the research outlined in the original proposal has progressed in a steady and productive manner as expected for this first year of funding. To delineate the accomplishments to date, the tasks outlined in the original Statement of Work for the first two years of the proposal are itemized below and a progress report for each task provided. Task 3 is not included as it will be completed in months 18 to 36 of this proposal.

Statement of Work

Task 1. To identify the Myc binding domain of TRRAP, a novel MycNTD-binding protein recently identified biochemically (months 1-18)

a. Complete. We have cloned overlapping fragments of TRRAP into the pBD prey vector using a PCR based approach and verify sequence is in-frame with the TUP-1 repressor domain

b. Complete. The yeast have been transformed with these TRRAP fusion proteins, and analyzed by western blot for equal expression of prey fusion proteins using our recently derived TUP-1 specific antibody

c. Complete. It has been established that the prey fusion proteins do not affect yeast viability in the presence or absence of 5-FOA

d. Complete. We have tested TRRAP fusion proteins for their interaction with wildtype MycNTD bait proteins, include MbI and MBII deletion and point mutants as well as specificity controls for MycNTD interaction, including Gal4E2F, Gal4VP16, etc.

e. In progress. The region of TRRAP required for interaction with Myc has been further refined using smaller fragments of TRRAP in the RTA. We are presently identifying the specific amino acids essential for interaction with the Myc N-terminal domain using the RTA. Once this is complete the required residues will be mutated and re-test in steps a-d above until a Myc-binding motif is identified. For details, please see Figure 1.

f. Yet to do. Search the sequence database for other polypeptides encoding this motif

g. Yet to do. If such proteins are identified, their cDNA will be cloned and tested for Myc interaction using the RTA

h. Yet to do. Additional proteins identified by this approach will be further characterized as stated in Task 2g-k

Task 2. To use the RTA to identify MycNTD-binding proteins that are essential for Myc to transform mammary epithelial cells (months 1-24)

a. Complete. The cDNA libraries have both been prepared in pBD prey vector with mRNA derived first from asynchronous MCF-7 cells and then from primary breast carcinoma samples. The former was constructed as a directional library while the latter was prepared as a non-directional library.

b. Complete. Both cDNA libraries have been screened such that all mRNA in the cells is represented in the library screen. The MCF-7 library is superior to the primary breast library and will be used for subsequent analyses.

c. Complete. Pick and streak resultant colonies and retest for specificity of interaction on selection medium +/- 5-FOA +/- methionine to induce prey expression as well as a master plate to retain yeast for future work (see Figure 2).

d. Complete. Those colonies that show interaction with MycNTD in a prey fusion specific manner will be processed further. To isolate prey, high-quality plasmid DNA will be isolated by Quiagen or Clonetech prep and then transformed by electroporation into electro-competent DH5alpha cells.

e. In progress. Resultant mini-prep prey vector DNA will be retested for functional interaction against the MycNTD using the RTA

f. In progress. Those that repeat will be tested for interaction with MycNTD containing deletion and point mutations in MbI and MbII.

g. In progress. Those that are dependent upon these highly conserved regions will undergo nucleotide sequencing

h. Yet to do. These will be analyzed for known and novel cDNAs and interaction further confirmed in vitro by GST fusions, in vivo by co-immunoprecipitation of ectopically

5

expressed and tagged proteins and finally by in vivo co-immunoprecipitation of endogenous proteins.

i. Yet to do. Ectopic expression of the tagged protein will be analyzed by immunofluorescence to determine subcellular localization relative to Myc. For the latter excellent antibody reagents are available.

j. Yet to do. Myc-binding proteins will be tested for their ability to alter Myc function. These assays will include Myc autosuppression, apoptosis, transformation, etc. These assays are well-established in the lab

k. Yet to do. The expression of Myc and the Myc-binding proteins will be analyzed for expression and activity in cell lines and primary tissue samples derived from normal and transformed mammary carcinomas

Key Research Accomplishments

•A region of TRRAP essential for interaction with Myc has been identified using the RTA. This Myc-binding domain of TRRAP and the Myc N-terminal domain are now under further analysis to define the precise amino acids required for this interaction.

•Two cDNA libraries have been constructed and screened to identify polypeptides that interact with the Myc N-terminus. Positive clones from the MCF-7 library are now being isolated, re-screened and sequenced.

Reportable Outcomes

None yet to report.

Conclusions

The work to date shows the expected progress and suggests the ultimate goal of the proposal will be accomplished during the tenure of this award. In the first year of funding specific objectives #1 and #2 have been initiated.

As described in the first specific aim, the RTA has been employed to identify a region of TRRAP which is required for binding to the Myc N-terminal domain. With this knowledge the precise amino acid residues essential for TRRAP:Myc interaction can be further identified using the RTA. This is presently in progress. Once the Myc-binding motif has been defined the research will progress in two directions. First, other proteins containing this motif will be identified using an in silico approach. With this class of Myc-binding proteins identified the nature of the polypeptides and their role in the control of cell proliferation will be further investigated. Second, inhibitors of the TRRAP:Myc interaction will be identified. With these lead compounds in hand novel therapeutics to block this interaction and Myc function as an oncogene will be further developed.

As outlined in the second specific aim, two cDNA libraries have been constructed and screened using the RTA to identify novel Myc binding proteins. Positive clones resulting from the MCF-7 library screen are presently being further characterized to ensure they are true Myc interactors and to reveal their identity through nucleotide sequencing. With these interactors in hand the molecular mechanism of action of Myc in the carcinogenic process will be further revealed. In addition, these clones will provide the essential experimental tools to develop additional therapeutics that will break Myc interactions that hold a key role in the transformation.

References

1. Hirst, M., Ho, C., Sabourin, L., Rudnicki, M., Penn, L., and Sadowski, I. (2001) A two-hybrid system for transactivator bait proteins. *Proc Natl Acad Sci U S A* 98, 8726-31.

2. McMahon, S. B., Van Buskirk, H. A., Dugan, K. A., Copeland, T. D., and Cole, M. D. (1998) The novel ATM-related protein TRRAP is an essential cofactor for the c- Myc and E2F oncoproteins. *Cell* 94, 363-74

3. Oster, S. K., Ho, C. S., Soucie, E. L., and Penn, L. Z. (2002) The myc oncogene: MarvelouslY Complex. *Adv Cancer Res* 84, 81-154
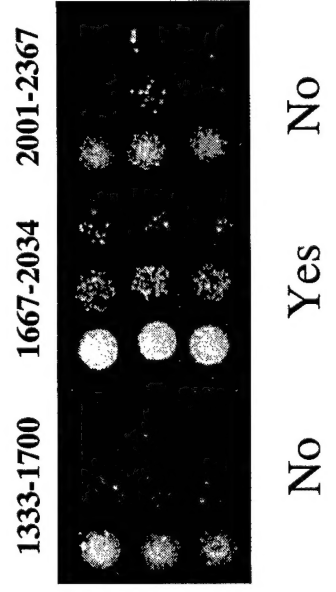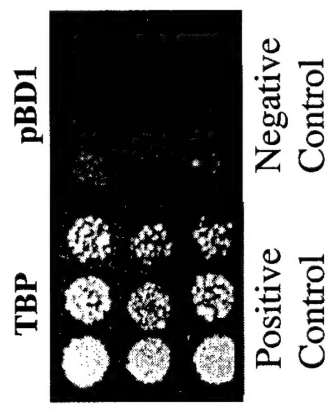
Appendices

Figure1

Figure 2

For details of the RTA, please appended manuscript:
Hirst, M., Ho, C., Sabourin, L., Rudnicki, M., Penn, L., and Sadowski, I. (2001) A two-hybrid system for transactivator bait proteins. *Proc Natl Acad Sci U S A* 98, 8726-31.

Figure 1: Fragments spanning TRRAP were assayed in the RTA and the region spanning amino acids 1667 to 2034 of TRRAP were shown to bind to the Myc NTD.
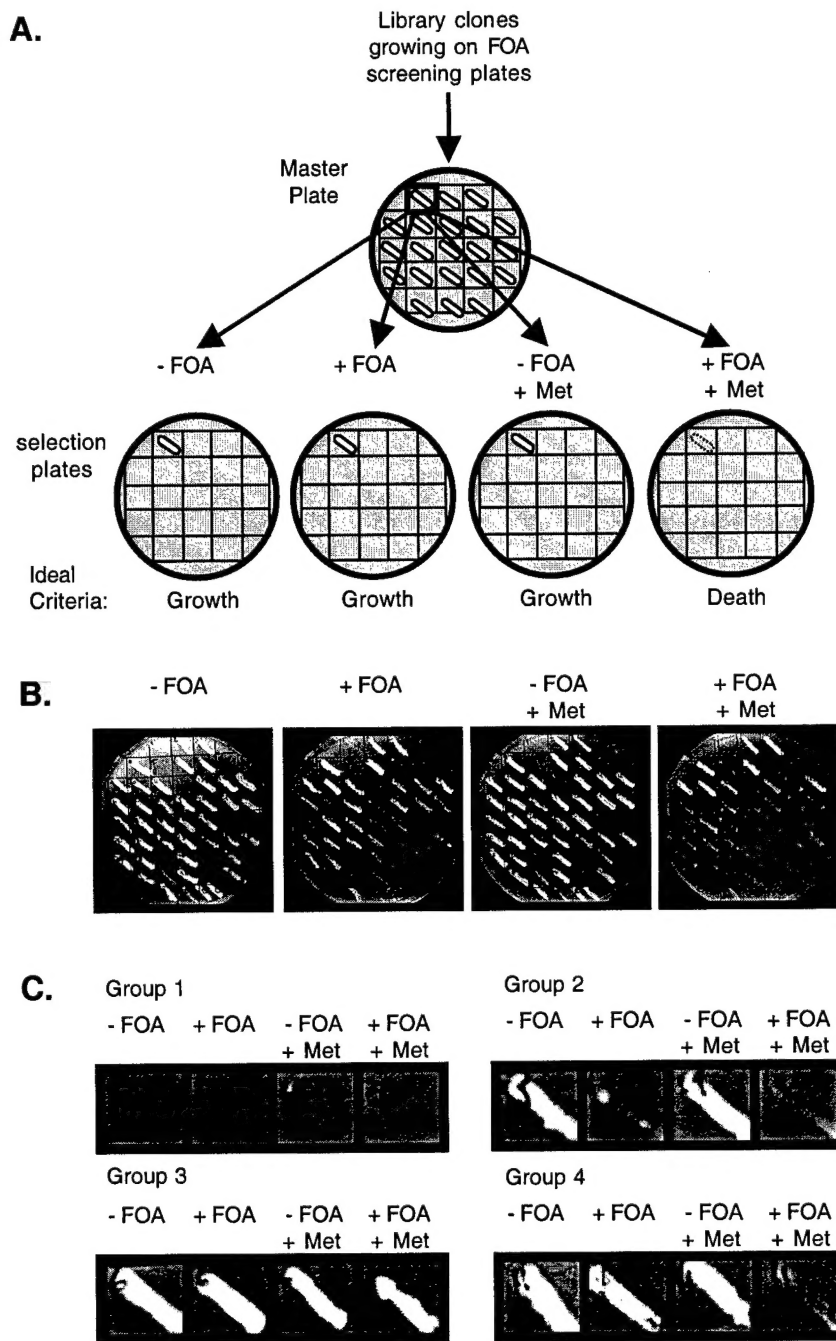
A.

Library clones
growing on FOA
screening plates

Master
Plate

- FOA + FOA - FOA + Met + FOA + Met

selection
plates

Ideal
Criteria: Growth Growth Growth Death

B.

- FOA + FOA - FOA + Met + FOA + Met

C.

Group 1
- FOA + FOA - FOA + Met + FOA + Met

Group 2
- FOA + FOA - FOA + Met + FOA + Met

Group 3
- FOA + FOA - FOA + Met + FOA + Met

Group 4
- FOA + FOA - FOA + Met + FOA + Met

Figure 2 Identification of novel Myc NTD-interacting protiens by library screen using RTA
(A) Schematic outline of library screen and selection criteria. FOA resistant colonies were collected and transferred from screening plates onto master plates. Library clones were then tested by directly transferring cells from master plates to a series of selective plates designed to test for specificity and recapitulation of growth phenotype on FOA. (B) A representative set of library selection plates. (C) Clones can be divided into 4 categories. Group 1 clones were viable when picked from original screening plates onto master plates, but were not viable when transferred from master plates to selection plates. Group 2 clones were viable on selection plates, but did not confer growth on FOA. Group 1 and 2 clones were not carried further. Group 3 clones were able to confer strong rescue on FOA, however, strong growth was also seen on +Met, suggesting that the growth on FOA could be non-specific. Group 4 clones exhibit the ideal selection criteria, rescue of growth on FOA and loss of this rescue when transferred to +Met indicating specificity of growth on FOA to the concurrent expression of the library TUP1-fusion.

A two-hybrid system for transactivator bait proteins

Martin Hirst*, Cynthia Ho†‡, Luc Sabourin§, Michael Rudnicki§, Linda Penn†‡, and Ivan Sadowski*¶

*Department of Biochemistry and Molecular Biology; University of British Columbia Vancouver, BC, Canada V6T 1Z3; †Department of Medical Biophysics and ‡Ontario Cancer Institute, University of Toronto, Toronto, ON, Canada M5G 2M9; and §Ottawa Hospital Research Institute, Ottawa, ON, Canada K1H 8L6

We describe a two-hybrid strategy for detection of interactions with transactivator proteins. This repressed transactivator (RTA) system employs the N-terminal repression domain of the yeast general repressor TUP1. TUP1-GAL80 fusion proteins, when coexpressed with GAL4, are shown to inhibit transcription of GAL4-dependent reporter genes. This effect requires the C-terminal 30 residues of GAL4, which are required for interaction with GAL80 *in vitro*. Furthermore, repression of *GAL* transcription by TUP1-GAL80 requires *SRB10*, demonstrating that the TUP1 repression domain, in the context of a two-hybrid interaction, functions by the same mechanism as endogenous TUP1. Using this strategy, we demonstrate interactions between the mammalian basic helix–loop–helix proteins MyoD and E12, and between c-Myc and Bin-1. We have also identified interacting clones from a TUP1-cDNA fusion expression library by using GAL4-VP16 as a bait fusion. These results demonstrate that RTA is generally applicable for identifying and characterizing interactions with transactivator proteins *in vivo*.

The yeast two-hybrid (1) and interaction-trap (2) systems are simple genetic strategies for detecting interactions between proteins *in vivo*. These techniques were developed consequent to the understanding that eukaryotic transcriptional activators have separable DNA-binding (DBD) and activation domains (AD) that function when fused to heterologous proteins (3). Interaction of an AD "prey" fusion with a DBD "bait" fusion protein produces a functional transactivator complex that activates reporter genes bearing upstream cis-elements for the DBD. One limitation of these strategies is that transcriptional activators cannot be used as baits. In this report we describe a modified strategy that addresses this problem.

The yeast general repressor protein TUP1 is recruited to specific promoters in a complex with the corepressor SSN6 by gene-specific DNA-binding proteins. TUP1/SSN6 complexes are recruited to glucose-repressed genes by MIG1, which binds the upstream repression sequence for glucose (URS$_G$) (4) (see Fig. 1A). Repression by SSN6/TUP1 is associated with chromatin reorganization of a number of genes including the *GAL4*, *GAL1*, *GAL10*, *SUC2*, and *MAT*a specific genes (5–8). This observation, coupled with the finding that the N terminus of TUP1 interacts directly with histone H3 and H4 tails (9), suggests that SSN6/TUP1 may repress transcription by organizing nucleosomes (10). Repression by SSN6/TUP1 is also affected by mutations to several RNA polymerase II holoenzyme-associated proteins, including the cyclin-dependent kinase/cyclin pair SRB10/SRB11 (11). This genetic relationship suggests that TUP1-mediated repression also involves modulation of RNA polymerase II holoenzyme function.

The N-terminal 200 residues of TUP1 are sufficient to cause repression when artificially recruited to a promoter as a LexA fusion (12). Based on this observation, we reasoned that the TUP1 repression domain should function in the context of a two-hybrid system to detect protein interactions with transcriptional activator bait fusions. We show that interaction of GAL4 DBD-activator bait fusion proteins with TUP1 repression domain (RD) prey fusions can be detected by repression of GAL4-dependent reporter gene transcription (see Fig. 1 B and C). We have called this two-hybrid variation the repressed transactivator (RTA) system, and we demonstrate that it is

capable of detecting interactions with transcriptional activator proteins *in vivo*.

Materials and Methods

Plasmids and Yeast Strains. Wild-type GAL4 was expressed from the *ADH1* promoter on the *HIS3*, 2μ vector pMA210 (13). The deletion derivatives pMA230 (1–848), pMA242 (1–238::768–881), pKW2 (1–238::768–848), pMA246 (1–239), and pMAB17 (1–147::B17) were as described (13, 14). Plasmid pJMH105 is a *TRP1* 2μ vector expressing the TUP1 RD from the *ADH1* promoter, which was constructed by cloning an EcoRI/NotI *TUP1* fragment, produced by amplification with primers MH50 (5′-GGCGAATTCGTATGACTGCCAGCGTTTCG) and MH51 (5′-GAGCGGCCGCTGCCACGGAAACCTGGG-GAGG), into YephaΔlac (15). *GAL80* was amplified by using primers MH54 (5′-GAGCGGCCGCTATGGACTACAACAA-GAG) and MH55 (5′-GAGCGGCCGCTTATAAACTATA-ATGCG), and cloned into YEpHAΔlac to create pJMH106 (*ADH1-GAL80*), or into pJMH105 to create pJMH107 (*ADH1-TUP1-GAL80*). Plasmid pJMH109 is a *LEU2* integrating vector containing a *GAL1-URA3* reporter gene, which was constructed by cloning an HindIII/BamHI *URA3* fragment, produced by amplification with the primers MH121 (5′-TTCTAAAGCT-TATGTCGAAAGCTACATATAAGGAACG) and MH122 (5′-TTATCGGATCCTTAGTTTTGCTGGCCGCATCTTC) into pAOGAL1, which is a *LEU2*-integrating *GAL1* expression plasmid. The pBD plasmids are *HIS3*, 2μ vectors that express TUP1 repression domain fusions from the *MET3* promoter, whereas the pG plasmids are derived from pPHO-GAL4 (16), and express GAL4 DBD fusions from the *PHO5* promoter on a *TRP1*, ARS/CEN backbone. Details of these vectors will be described elsewhere. The pG-Myc (1–262) transactivation domain was cloned into the SmaI site of pG1 (17) as an XhoI/SmaI fragment (made blunt). The pBD-BIN1 fusion was constructed by inserting a BamHI/BstXI Bin1 cDNA fragment (18) (made blunt) into the SmaI site of pBD2. Plasmid pGAL4-MyoD expressing GAL4-MyoD contains an EcoRI/HindIII DNA fragment consisting of the *ADH1* promoter and terminator expressing GAL4 (1–147) fused to mouse MyoD residues (1–319), cloned into Ycplac22 (19). Plasmid pRSTE425 expressing TUP1-mouse E12 (residues 274–444) from the *PGK* promoter/terminator (20), contains a BamHI/BglII E12 DNA fragment fused to the TUP1 RD on pRS425 (21). The TUP1-E12ΔH2 derivative produced from pRSTEΔH2 bears a deletion of E12 residues (376–444).

Yeast strains were as follows: YJMH1 (*MAT*α, gal4-542, gal80-538, ura3-52, his3-200, ade2-101, ade1, lys2-80, trp1-901, ara1, leu2-3,112, met, LEU2::GAL1-URA3 (pMH109));

YT6::171 (*MATα, gal4, gal80, ura3, his3, ade2, ade1, lys2, trp1, ara1, leu2, met, URA3::GAL1-LacZ*) (22); W303::131 [*MATa, ade2, his3, leu2, trp1, ura3, can1, URA3::GAL1-lacZ* (pRY131)] (23); H617::131 [*MATα, ade2, can1, his3, leu2, trp1, ura3, srb10::HIS3, URA3::GAL1-LACZ* (pRY131)] (24); MAV108 (*MATa, gal4, gal80, leu2, trp1, his3, SPAL10::URA3*) (25); and Y190 (*MATα, ura3-52, leu2-3,112 trp1-901, his3Δ200, ade2-101, gal4, gal80, cyh2^R, URA3::GAL1-lacZ, LYS2::GAL1-HIS3*) (1).

Growth and β-Galactosidase Assays. Growth assays were performed in strain YJMH1 (GAL4 baits) or MAV108 (GAL4-Myc baits) on synthetic dextrose medium (SD) lacking histidine and tryptophan, containing either 2% galactose and 2% raffinose (GAL/RAF) or 2% glucose (GLU) and 5-fluoroorotic acid (5-FOA) where indicated. Cells were suspended in water and normalized to an $OD_{600} = 1.0$ and were 10-fold serially diluted in water. Ten microliters of the dilutions were spotted onto selective agar plates and incubated for 3–5 days at 30°C. Cells for β-galactosidase assays were grown in SD to an $OD_{600} = 1.0$; activity was measured from extracts prepared by lysis with glass beads (16), and are an average of at least three independent determinations.

Construction of the TUP1-MCF-7 cDNA Fusion Library. RNA was purified from 5×10^7 exponentially growing MCF-7 cells by using QuickPrep mRNA purification kit (Amersham Pharmacia; catalog no. 27-9254-01) and twice purified on Oligo(dT)-cellulose spin columns. cDNA was synthesized by using the Timesaver cDNA synthesis kit (Amersham Pharmacia; catalog no. 27-9262-01) with modifications to the protocol to produce directional cDNAs. In brief, poly(A)$^+$ RNA was subjected to random primed cDNA synthesis using a specialized direct random hexamer (N_6CC). cDNAs were methylated after second strand synthesis, and excess primers were removed in a spin column. Methylated cDNAs were ligated to annealed directional adaptors (AATTCGTCGACGGAT/ATCCGTCGACG). The ligated products were then phosphorylated, digested with *Bam*HI to produce cDNAs with 5' *Eco*RI and 3' *Bam*HI sticky ends, and then ligated into appropriately digested pBD1. Ligated DNA was electroporated into ElectroMAX DH10B electrocompetent cells (GIBCO/BRL, Bethesda; catalog no. 18290-015).

GAL4-VP16AD RTA Screen. Yeast strain MAV108 bearing plasmid pY1VP16 (26) expressing GAL4-VP16AD (411–490) was transformed with the pBD-MCF-7 cDNA library and allowed to form colonies on SD lacking histidine and tryptophan. About 6.0×10^6 transformants were scraped, resuspended in 50% glycerol, snap frozen in liquid nitrogen, and stored at −70°C. Aliquots were thawed and plated on SD lacking histidine, tryptophan, and methionine and containing 0.1% 5-FOA at a density of 1.2×10^6 colony-forming units per 15-cm plate. Colonies were picked after 5–7 days growth at 30°C, and expanded in liquid SD lacking histidine. Cells were harvested by centrifugation, resuspended in 100 µl of Y-PER (Pierce; catalog no. 78990), and incubated at room temperature for 20 min. DNA was prepared from the lysates by sequential addition of solution II and solution III as with the standard alkaline lysis miniprep protocol (27). cDNA inserts were amplified by PCR using the vector-specific primers oKL1 (5'-TTGCCTGTGGTGTCCTCAAAC) and oKL2 (5'-TGACCAACCTCTGGCGAAG), purified by using QIAquick columns (Qiagen, Chatsworth, CA; catalog no. 28106), and sequenced with oKL1. Insert templates for production of *in vitro*-translated protein were produced by PCR with oligo oIS923 (5'-CCCTCGAG<u>TAATACGACTCACTATAGG</u>GAGC-CACCATGCAGCAACCACCTCCCCAGGTTTCCGTGGC-AG-3') containing a T7 promoter tag (underlined), and oKL2. [^{35}S]Methionine-labeled *in vitro*-translated protein was produced by coupled transcription/translation, directly from the
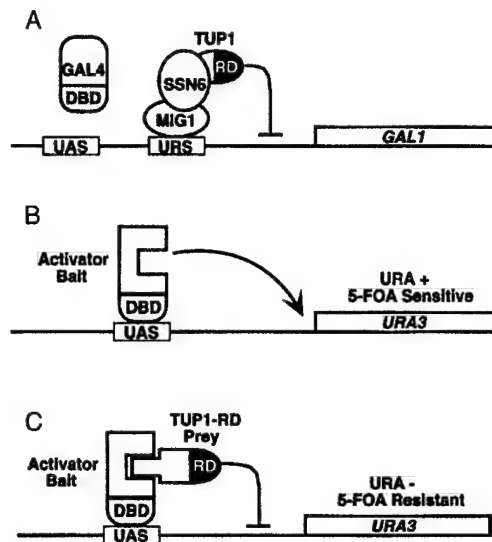

Fig. 1. Rationale for the repressed transactivtor two-hybrid system. (*A*) Representation of the *GAL1* gene in glucose-grown cells, indicating the binding sites for GAL4 (UAS), and MIG$_1$ (URS). MIG$_1$ recruits the general repressors SSN6 and TUP1. The N-terminal RD of TUP1 is indicated in black. (*B*) Activation of a *GAL1-URA3* reporter gene by a GAL4 DBD-activator fusion protein (activator bait) in *ura3*$^-$ yeast gives a URA$^+$ phenotype, and causes sensitivity to 5-FOA. (*C*) Interaction between a TUP1 RD fusion (TUP1-RD prey) with the activator bait causes repression of transcription, resulting in 5-FOA resistance.

PCR template, by using the TNT T7 Quick system (Promega; catalog no. L5540). Interaction of labeled insert-encoded proteins with recombinant glutathione *S*-transferase (GST)-VP16 was performed as described previously (28, 29).

Results

The TUP1 N Terminus Represses *GAL* Transcription When Fused to GAL80. The N terminus of TUP1 causes repression when fused directly to LexA (12). We examined whether this repression domain was capable of functioning when recruited to DNA by a specific protein–protein interaction as a prey fusion-protein (Fig. 1*C*). We imagined that such an interaction would mimic the natural function of TUP1, which is normally recruited to specific promoters by DNA-binding proteins as a complex with SSN6 (Fig. 1*A*). Repression of transcription can be genetically detected by using counterselectable reporter genes such as *URA3* (30). Expression of *URA3* allows growth of yeast in the absence of uracil (URA$^+$), but also renders them sensitive to 5-FOA, which is converted into a toxic metabolite by the *URA3* gene product (Fig. 1*B*). Repression of *URA3* can be detected by the ability to grow in the presence of 5-FOA (Fig. 1*C*).

We examined the feasibility of the RTA system by using the well-defined interaction between GAL4 and its regulator GAL80. GAL4 expressed on its own from the *ADH1* promoter in a strain bearing a *GAL1-URA3* reporter gene causes activation of transcription and renders the yeast sensitive to 5-FOA (Fig. 2, line 1). Expression of GAL80 from the *ADH1* promoter in the same strain causes slight inhibition of GAL4, allowing infrequent formation of colonies on 5-FOA (Fig. 2, line 2), but expression of the TUP1 RD on its own has no effect on activation by GAL4 (Fig. 2, line 3). In contrast, coexpression of a TUP1 RD-GAL80 fusion with GAL4 allows growth on 5-FOA (Fig. 2, line 4). We observed this result whether the yeast were grown on glucose- (not shown), or galactose-
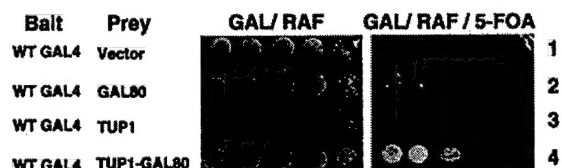
Fig. 2. TUP1-GAL80 represses GAL transcription under inducing conditions. Yeast strain YJMH1 was cotransformed with pMA210 expressing wild-type (WT) GAL4 (bait), and one of the following: pJMH106, expressing GAL80 (line 2); pJMH105, expressing TUP1 RD (line 3); pJMH107, expressing TUP1-GAL80 (line 4); or YephalΔlac (line 1). Equivalent numbers of cells were spotted, in 10-fold serial dilutions (from left to right), onto selective media containing galactose and raffinose without 5-FOA (GAL/RAF), or with 0.1% 5-FOA (GAL/RAF/5-FOA).

containing plates (Fig. 2). This result demonstrates that the TUP1 RD inhibits activation by GAL4 when fused to the N terminus of GAL80.

SRB10 Is Required for Repression by the N Terminus of TUP1. SRB10 has been shown to be required for at least part of the repressive effect of the TUP1 RD *in vivo* (11, 31). To determine whether the TUP1 RD inhibits transcription by its normal repressive mechanism when fused to GAL80, we examined whether *SRB10* was required for repression of GAL4-dependent reporter gene expression by TUP1-GAL80. For these experiments we used a *GAL1-lacZ* reporter gene (Fig. 3). Consistent with the results of Fig. 2, we found that TUP1-GAL80 inhibited transcriptional activation by GAL4 in wild-type *SRB10* yeast (Fig. 3, TUP1-GAL80). GAL80, expressed on its own, caused only slight inhibition of GAL4 (Fig. 3, GAL80), whereas the TUP1 RD had no effect on its own (Fig. 3, TUP1). In contrast, we found that TUP1-GAL80 was incapable of inhibiting activation of *GAL1-lacZ* reporter gene expression in an srb10 disruption strain (Fig. 3, srb10 TUP1-GAL80). This result supports the conclusion that the TUP1 RD fused to GAL80 causes repression through its normal function, rather than by simply augmenting an inhibitory effect of GAL80.

Repression of GAL Transcription by TUP1-GAL80 Requires the GAL4 C-Terminal 30 Residues. GAL4 contains two regions that activate transcription when fused individually to the DBD, known as activating region 1 and activating region 2 (AR1 and AR2, respectively; see Fig. 4A) (13). AR2 overlaps a region required for interaction with GAL80 (14). Deletion of the C-terminal 30 residues produces a constitutive activator that is incapable of efficient interaction with GAL80 (14). To determine whether repression of transcription by TUP1-GAL80 reflected interaction with the C-terminal 30 residues of GAL4, we examined a series of GAL4 deletion/fusion derivatives. We found that only those GAL4 derivatives bearing the C-terminal 30 residues allowed survival of yeast on 5-FOA when coexpressed with TUP1-GAL80 (Fig. 4B, lines 1 and 3). Thus, yeast expressing GAL4(1–848) coexpressed with TUP1-GAL80 could not grow on 5-FOA (Fig. 4, line 2), indicating that deletion of the C-terminal 30 residues prevents repression of the *URA3* reporter. Similarly, a derivative bearing both AR1 and AR2 but lacking the central region [GAL4(1–238::768–881)] was inhibited by TUP1-GAL80 (Fig. 3, line 3), but a comparable derivative lacking the C-terminal 30 residues was not [GAL4(1–238::768–848), Fig. 3, line 4]. GAL4 DBD derivatives bearing only AR1 (Fig. 3, line 5), or the B17 activating peptide (32) (Fig. 3, line 6) were also insensitive to inhibition by TUP1-GAL80. When expressed in the absence of TUP1-GAL80, all of the GAL4 derivatives in Fig. 4A activate the
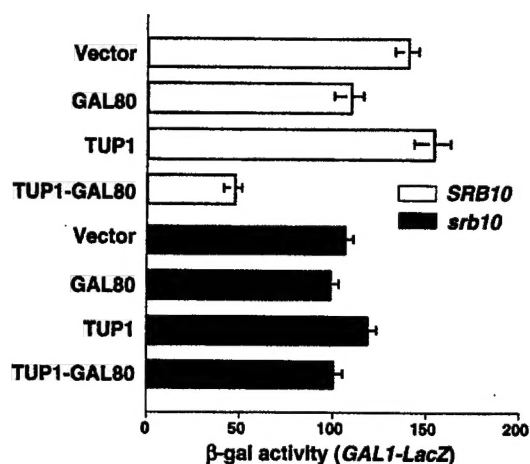

Fig. 3. Repression of GAL transcription by TUP1-GAL80 requires *SRB10*. Yeast strains W303:131 (*SRB10*, open bars), and H617:131 (*srb10*, black bars) were transformed with a vector control (YephalΔlac) (Vector) or plasmids expressing GAL80 (pJMH106), TUP1 (pJMH105), or TUP1-GAL80 (pJMH107). Yeast was grown to mid-log arithmic phase in selective medium containing galactose, and expression of the *GAL1-LacZ* reporter gene was assayed by measuring β-galactosidase activity.

GAL1-URA3 reporter gene to allow growth on URA⁻ plates, demonstrating that all of the derivatives are efficient activators (Fig. 4C). These results indicate that repression by TUP1-GAL80 requires the normal site of interaction between GAL4 and GAL80.

RTA Detects Interactions with Mammalian Transactivator Proteins. We also examined whether the TUP1 RD could be used to detect interactions with mammalian transactivator bait fusions. MyoD is a basic helix–loop–helix (bHLH) family member involved in skeletal muscle development (33). MyoD forms heterodimers with the ubiquitously expressed bHLH protein E12 (34). When expressed in yeast as a fusion to the GAL4 DBD, MyoD causes strong activation of a *GAL1-lacZ* reporter gene (Fig. 5A). Coexpression of GAL4-MyoD with the TUP1 RD on its own did not affect reporter gene expression (Fig. 5A, TUP1), but coexpression of a TUP1-E12 fusion protein repressed *GAL1-lacZ* transcription (Fig. 5A, TUP1-E12). In contrast, expression of a TUP1-E12 fusion bearing a deletion of the HLH helix 2, which is known to be required for interaction with MyoD *in vitro* (35), had no effect on activation by GAL4-MyoD (TUP1-E12ΔH2). Similar to the results presented in Figs. 2 and 4, we also found that coexpression of TUP1-E12 with GAL4-MyoD in yeast bearing a *GAL1-URA3* reporter allowed growth on plates containing 5-FOA (not shown). These results demonstrate that the specific interaction between MyoD and E12 can be detected *in vivo* by using RTA.

We also tested interaction between the protooncogene product c-Myc and the putative tumor suppressor protein Bin1, which has been shown to interact with the N-terminal c-Myc transactivation domain (36). The c-Myc N terminus fused to GAL4 DBD causes activation of transcription of GAL4-dependent reporter genes, and causes killing of yeast bearing a *GAL1-URA3* reporter gene on 5-FOA (Fig. 5B, line 3). Coexpression of a TUP1 RD-Bin1 fusion with GAL4-Myc allowed survival on 5-FOA (Fig. 5B, line 4), indicating repression of the *GAL1-URA3* reporter, and demonstrates specific interaction between c-Myc and Bin1 *in vivo*.
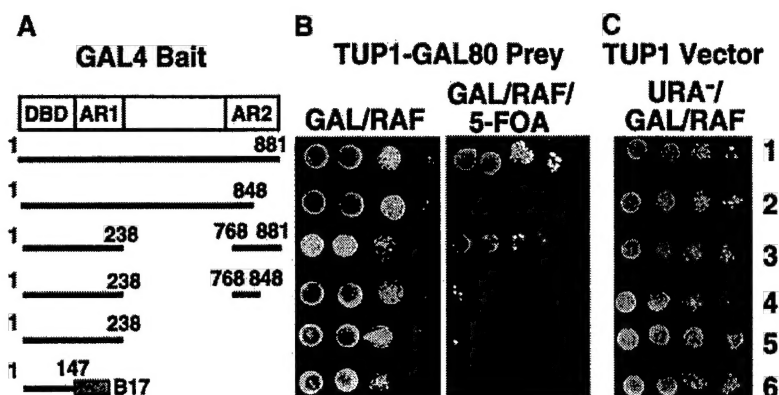
A
GAL4 Bait

B
TUP1-GAL80 Prey

C
TUP1 Vector

Fig. 4. Repression of *GAL* transcription by TUP1-GAL80 requires the GAL4 C-terminal 30 amino acids. (*A*) Fusion derivatives and deletions of GAL4. (*B*) Yeast strain yJMH1 was cotransformed with pJMH107 expressing TUP1-GAL80 and plasmids expressing the GAL4 deletion/fusion derivatives indicated in *A*. Cells were spotted, in 10-fold serial dilutions (from left to right) on selective plates containing galactose and raffinose without 5-FOA (*B*, GAL/RAF), or with 0.1% 5-FOA (*B*, GAL/RAF/5-FOA). (*C*) Yeast cotransformed with GAL4 expression plasmids (indicated in *A*) and pJMH105 were spotted as above on minimal selective plates lacking uracil (URA⁻/GAL/RAF), to assay activation of the *GAL1-URA3* reporter.

Identification of VP16-Interacting Proteins by Using RTA.

To determine whether RTA could be used to screen for novel interactions with a transactivator, a cDNA expression library was screened for proteins that interact with the VP16 activation domain. Yeast strain MAV108 (25), bearing a *GAL*$_{UAS}$-*URA3* reporter gene and a GAL4-VP16 expression plasmid, was cotransformed with a TUP1 RD-cDNA fusion expression library. Interacting clones were selected for repression of

Fig. 5. RTA detects interactions with mammalian transactivators. (*A*) Yeast strain Y190 was cotransformed with pGAL4-MyoD and a vector control (pRS425), pRST425 expressing TUP1-RD, pRSTE425 expressing TUP1-E12, or pRSTE425ΔH2 expressing TUP1-E12 bearing a deletion of helix 2 (TUP1-E12ΔH2). Yeast was grown to mid-log arithmic phase in selective medium containing glucose, and expression of the *GAL1-lacZ* reporter gene was assayed by measuring β-galactosidase activity. (*B*) Yeast strain MAV108 was transformed with the bait vector control plasmid (pG1), or pG-Myc(1–262) expressing GAL4-Myc, in combination with the prey vector control plasmid pBD1 (TUP1) or a pBD-BIN expressing TUP1-BIN1. Cells were spotted, in 10-fold serial dilutions (from left to right) on selective plates containing glucose without 5-FOA (GLU), or with 0.05% 5-FOA (GLU/5-FOA).

URA3 by growth on plates containing 0.1% 5-FOA. Sequences were obtained for 30 independent 5-FOA-resistant clones that contained in-frame TUP1-cDNA fusions (Table 1). Multiple isolates were obtained for several of the clones, indicating that the screen may have neared saturation (Table 1). To determine whether any of the clones represented direct interactions with the VP16 activation domain, we assayed *in vitro* binding of [³⁵S]methionine-labeled insert-encoded protein to recombinant GST and GST-VP16 (data not shown). We found that proteins produced by the BCL7B, HSHZF4, KIAA0710, and TAF$_{II}$68 inserts interacted specifically with GST-VP16 (Table 1). In contrast, the HSPA5 and DDX5 proteins interacted equally well with GST and GST-VP16, and therefore likely represent nonspecific interactions. The Pol2RA insert-encoded protein did not interact with GST or GST-VP16, indicating that it may represent an indirect interaction with VP16 *in vivo*. Some of the additional clones may also represent false positives (kynureninase, UDP-glucose dehydrogenase). In summary, these results demonstrate that RTA can be used to screen for novel proteins that interact with transactivators. Our screen using RTA identified specific clones at a proportion comparable to screens using the standard two-hybrid and interaction trap systems.

Discussion

We demonstrate that the repression domain of TUP1 can function when recruited to promoters by specific interactions between fusion proteins. The TUP1 RD fused to GAL80 causes repression of GAL4-dependent reporter genes. Repression by TUP1-GAL80 requires the C-terminal 30 residues of GAL4, which are known to interact with GAL80 *in vitro*, and are necessary for normal inhibition by GAL80 *in vivo*. Furthermore, repression by TUP-GAL80 requires *SRB10*, indicating that the TUP1 RD inhibits transcription through its normal function in the context of the two-hybrid interaction. We show that this property of the TUP1 RD can be exploited for detecting interactions with transactivator proteins *in vivo*. Protein interactions with two different mammalian transcription factors, MyoD and c-Myc, were detected by using RTA. We have also performed a pilot scale two-hybrid screen using GAL4-VP16 as a bait to identify several interacting proteins not previously described. These results demonstrate the usefulness of RTA for characterizing interactions with transactivators *in vivo*.

Proteins that normally function to activate transcription, or proteins that artificially activate when fused to a DBD, cannot be used as baits in the standard two-hybrid or interaction-trap systems unless the regions that cause activation are deleted.
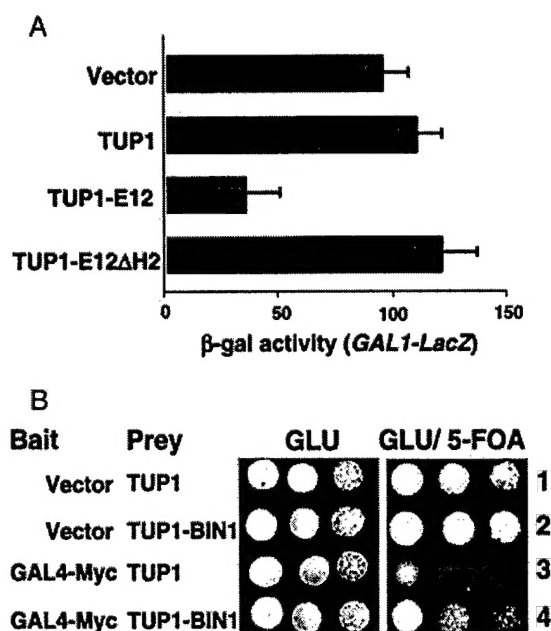
Table 1. Identification of VP16-interacting proteins by using RTA

Sequence	Clones*	In vitro†	Function/notes	Ref.
bA155N3.1	4	I	Novel protein similar to KIAA0694	37
BCL7B	3	+	Rearranged in B cell lymphoma, novel conserved gene family	38
DDX5	1	NS	DEAD-box helicase, 68 kDa	39
HSHZF4	1	+	Krüppel-related zinc finger protein	40
KIAA0710	1	+	Unknown function	37
Pol2RA	1	−	RNA polymerase II 220-kDa α-subunit	41
hSAN	1	I	Similar to *Drosophila melanogaster* separation anxiety protein, AK023256	
TAF$_{II}$68	1	+	68-kDa TBP-associated factor	42
28S	1	ND	28S Ribosomal RNA	
HSPA5	2	NS	BiP heat shock protein	43
Kynureninase	1	ND	L-Kynurenine hydrolase	44
UGDH	1	ND	UDP-glucose dehydrogenase	45
ESTs	3	ND	Uncharacterized EST sequences	
Unannotated	9	ND	Sequences from unannotated genomic DNA	

BiP, immunoglobulin light chain binding protein; EST, expressed sequence tag.
*Number of independent isolates.
†I, inconclusive; +, *in vitro* interaction; −, no *in vitro* interaction; NS, nonspecific *in vitro* interaction; ND, not determined.

This problem needed to be addressed because critical interactions between transcription factors and their regulators are often mediated by the same regions that cause transcriptional activation. This is illustrated in the results presented here. Interaction of GAL80 with GAL4 requires the C-terminal 30 residues of GAL4. This region is also necessary for efficient activation of transcription, and is known to contact several general initiation factors (28, 46). In addition, interaction between the putative tumor suppressor protein Bin1 requires the N-terminal transactivation domain of c-Myc (47). Our experiments demonstrate that the RTA system is useful for genetically detecting interactions with transactivation domains. However, the assay is not dependent on direct interaction of the TUP1-prey fusion with the activation domain of the activator bait, because we also demonstrate interaction between MyoD and its heterodimer partner E12. This interaction occurs through helix 2 of the bHLH motif (48), which is not directly responsible for transactivation.

In our screen with GAL4-VP16 as a bait fusion, we identified several previously undescribed interacting proteins, including BCL7B, HZF4, and TAF$_{II}$68. These appear to be direct interactions, as protein produced by *in vitro* transcription/translation of the cDNA inserts bound GST-VP16 in pull-down assays (data not shown, and Table 1). The significance of these interactions for activation by VP16, or replication of HSV-1, will require confirmation and characterization of the interactions *in vivo*. Interaction of an activator with TAF$_{II}$68 has not been demonstrated previously, although several viral activators are known to interact with TAF$_{II}$250 (49–51), and TAF$_{II}$40 (52). Surprisingly, in our screen, we did not identify general transcription factors (GTFs) that are known transcriptional

activation targets for VP16 *in vitro* (52–55). It is possible that these interactions are too weak to be identified at the 5-FOA concentration used, or some TUP1-GTF fusions may be toxic in yeast due to nonspecific recruitment to promoters. One clone representing the largest RNA polymerase II subunit (Pol2RA) was recovered in the screen, but we did not detect interaction of the protein with VP16 *in vitro* (not shown). This result indicates that, as with the standard two-hybrid and interaction-trap systems, some interactions between the bait and prey fusions in RTA may be mediated through complexes with one or more yeast proteins.

Several other two-hybrid systems can be used with bait proteins that activate transcription. The Sos- and Ras-recruitment two-hybrid strategies rely on interaction between the bait and prey fusions for recruitment of CDC25 or Ras to the plasma membrane, allowing growth at the nonpermissive temperatures in *cdc25* or *ras* temperature-sensitive strains (56, 57). Additionally, an RNA polymerase III-based system has been developed that utilizes the activating protein τ138 (58). The advantage of RTA over these strategies is that interactions with transactivator baits can be assayed in the context of their normal function—when they are localized to the nucleus, bound to a promoter, and activating RNA polymerase II-dependent transcription. We expect that the RTA system will be useful for characterizing interactions between transcriptional activator proteins and their regulatory molecules and coactivators, as well as identifying novel interactions involved in transcriptional regulation.

1. Fields, S. & Song, O. (1989) *Nature (London)* **340**, 245–246.
2. Golemis, E., Gyuris, J. & Brent, R. (1994) in *Current Protocols in Molecular Biology* (Wiley, New York), Vol. 13. pp. 20.1.1–2.1.40.
3. Brent, R. & Ptashne, M. (1985) *Cell* **43**, 729–736.
4. Nehlin, J. O., Carlberg, M. & Ronne, H. (1991) *EMBO J.* **10**, 3373–3377.
5. Bu, Y. & Schmidt, M. C. (1998) *Nucleic Acids Res.* **26**, 1002–1009.
6. Cooper, J. P., Roth, S. Y. & Simpson, R. T. (1994) *Genes Dev.* **8**, 1400–1410.
7. Frolova, E., Johnston, M. & Majors, J. (1999) *Nucleic Acids Res.* **27**, 1350–1358.
8. Gavin, I. M. & Simpson, R. T. (1997) *EMBO J.* **16**, 6263–6271.
9. Edmondson, D. G., Smith, M. M. & Roth, S. Y. (1996) *Genes Dev.* **10**, 1247–1259.
10. Huang, L., Zhang, W. & Roth, S. Y. (1997) *Mol. Cell. Biol.* **17**, 6555–6562.
11. Kuchin, S. & Carlson, M. (1998) *Mol. Cell. Biol.* **18**, 1163–1171.
12. Tzamarias, D. & Struhl, K. (1994) *Nature (London)* **369**, 758–761.
13. Ma, J. & Ptashne, M. (1987) *Cell* **48**, 847–853.
14. Ma, J. & Ptashne, M. (1987) *Cell* **50**, 137–142.
15. Hung, W., Olson, K. A., Breitkreutz, A. & Sadowski, I. (1997) *Eur. J. Biochem.* **245**, 241–251.
16. Stone, G. & Sadowski, I. (1993) *EMBO J.* **12**, 1375–1385.
17. Kato, J. G., Barrett, J., Villa-Garcia, M. & Dang, C. V. (1990) *Mol. Cell. Biol.* **10**, 5914–5920.
18. Wechsler-Reya, R., Sakamuro, D., Zhang, J., Duhadaway, J. & Prendergast, G. C. (1997) *J. Biol. Chem.* **272**, 31453–31458.
19. Gietz, R. D. & Sugino, A. (1988) *Gene* **74**, 527–534.
20. Masuda, Y., Park, S. M., Ohkuma, M., Ohta, A. & Takagi, M. (1994) *Curr. Genet.* **5**, 412–417.
21. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. & Hieter, P. (1992) *Gene* **110**, 119–122.
22. Sadowski, I., Niedbala, D., Wood, K. & Ptashne, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10510–10514.

23. Thomas, B. J. & Rothstein, R. (1989) *Cell* **56**, 619–630.
24. Balciunas, D. & Ronne, H. (1995) *Nucleic Acids Res.* **23**, 4421–4425.
25. Vidal, M., Brachmann, R. K., Fattaey, A., Harlow, E. & Boeke, J. D. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 10315–10320.
26. Sadowski, I., Bell, B., Broad, P. & Hollis, M. (1992) *Gene* **118**, 137–141.
27. Birnboim, H. C. (1983) *Methods Enzymol.* **100**, 243–255.
28. Melcher, K. & Johnston, S. A. (1995) *Mol. Cell. Biol.* **15**, 2839–2848.
29. Barlev, N. A., Candau, R., Wang, L., Darpino, P., Silverman, N. & Berger, S. L. (1995) *J. Biol. Chem.* **270**, 19337–19344.
30. Boeke, J. D., Trueheart, J., Natsoulis, G. & Fink, G. R. (1987) *Methods Enzymol.* **154**, 164–175.
31. Lee, M., Chatterjee, S. & Struhl, K. (2000) *Genetics* **155**, 1535–1542.
32. Ma, J. & Ptashne, M. (1987) *Cell* **51**, 113–119.
33. Megeney, L. A. & Rudnicki, M. A. (1995) *Biochem. Cell. Biol.* **73**, 723–732.
34. Czernik, P. J., Peterson, C. A. & Hurlburt, B. K. (1996) *J. Biol. Chem.* **271**, 9141–9149.
35. Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., *et al.* (1989) *Cell* **58**, 537–544.
36. Sakamuro, D., Elliott, K. J., Wechsler-Reya, R. & Prendergast, G. C. (1996) *Nat. Genet.* **14**, 69–77.
37. Ishikawa, K., Nagase, T., Suyama, M., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N. & Ohara, O. (1998) *DNA Res.* **5**, 169–176.
38. Jadayel, D. M., Osborne, L. R., Coignet, L. J., Zani, V. J., Tsui, L. C., Scherer, S. W. & Dyer, M. J. (1998) *Gene* **224**, 35–44.
39. Rossler, O. G., Hloch, P., Schutz, N., Weitzenegger, T. & Stahl, H. (2000) *Nucleic Acids Res.* **28**, 932–939.
40. Abrink, M., Aveskogh, M. & Hellman, L. (1995) *DNA Cell Biol* **14**, 125–136.
41. Cho, K. W., Khalili, K., Zandomeni, R. & Weinmann, R. (1985) *J. Biol. Chem.* **260**, 15204–15210.
42. Bertolotti, A., Lutz, Y., Heard, D. J., Chambon, P. & Tora, L. (1996) *EMBO J.* **15**, 5022–5031.
43. Koh, S. S., Ansari, A. Z., Ptashne, M. & Young, R. A. (1998) *Mol. Cell* **1**, 895–904.
44. Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Staller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M. & Prendergast, G. C. (1999) *Oncogene* **18**, 3564–3573.
45. Shirakata, M. & Paterson, B. M. (1995) *EMBO J.* **14**, 1766–1772.
46. Carrozza, M. J. & DeLuca, N. A. (1996) *Mol. Cell. Biol.* **16**, 3085–3093.
47. Geisberg, J. V., Chen, J. L. & Ricciardi, R. P. (1995) *Mol. Cell. Biol.* **15**, 6283–6290.
48. Weissman, J. D., Brown, J. A., Howcroft, T. K., Hwang, J., Chawla, A., Roche, P. A., Schiltz, L., Nakatani, Y. & Singer, D. S. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 11601–11606.
49. Goodrich, J. A., Hoey, T., Thut, C. J., Admon, A. & Tjian, R. (1993) *Cell* **75**, 519–530.
50. Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, J. L., Triezenberg, S. J., Reinberg, D., Flores, O., Ingles, C. J., *et al.* (1994) *Mol. Cell. Biol.* **14**, 7013–7024.
51. Lin, Y. S., Ha, I., Maldonado, E., Reinberg, D. & Green, M. R. (1991) *Nature (London)* **353**, 569–571.
52. Kobayashi, N., Horn, P. J., Sullivan, S. M., Triezenberg, S. J., Boyer, T. G. & Berk, A. J. (1998) *Mol. Cell. Biol.* **18**, 4023–4031.
53. Broder, Y. C., Katz, S. & Aronheim, A. (1998) *Curr. Biol.* **8**, 1121–1124.
54. Aronheim, A., Zandi, E., Hennemann, H., Elledge, S. J. & Karin, M. (1997) *Mol. Cell. Biol.* **17**, 3094–3102.
55. Marsolier, M. C., Prioleau, M. N. & Sentenac, A. (1997) *J. Mol. Biol.* **268**, 243–249.

GENETICS